RESEARCH ARTICLE

Association of Methylation of the RAR-β Gene with Cigarette Smoking in Non-Small Cell Lung Cancer with Southern-central Chinese Population

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Abstract

Pathogenesis of lung cancer is a complicated biological process including multiple genetic and epigenetic changes. Since cigarette smoking is confirmed as the most main risk factor of non-small cell lung cancer (NSCLC), the aim of this study was to determine whether tobacco exposure plays a role in gene methylation. Methylation of the RAR-β gene were detected using methylation-specific polymerase chain reaction in DNA from 167 newly diagnosed cases with NSCLC and corresponding 105 controls. A significant statistical association was found in the detection rate of the promoter methylation of RAR-β gene between NSCLC and controls (x^2=166.01; p<0.01), and hypermethylation of the RAR-β gene was significantly associated with smoking status (p=0.038, p<0.05). No relationship was found between RAR-β gene methylation and pathologic staging including clinical stage, cell type, gender and drinking (p>0.05), and the methylation of RAR-β gene rate of NSCLC was slightly higher in stages III+IV (80.0%) than in I+II (70.8%). Similar results were obtained for methylation of the RAR-β gene between squamous cell carcinoma (77.9%) and other cell type lung cancer (73.9%). These results showed that the frequency of methylation increased gradually with the development of clinical stage in smoking-associated lung cancer patients, and tobacco smoke may be play a potential role in RAR-β gene methylation in the early pathogenesis and process in lung cancer, particularly squamous cell carcinoma. Aberrant promoter methylation is considered to be a promising marker of previous carcinogen exposure and cancer risk.

Keywords: Methylation - non-small cell lung cancer - RARβ gene - cigarette exposure

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Introduction

Lung cancer is the leading cause of cancer deaths in China and worldwide (Siegel et al., 2013; Cheng et al., 2012). Five-year survival rate of lung cancer patients is less than or equal 15%, Non-small cell lung cancer accounts for 80% of all lung cancer. An important reason for the patients is that lung cancer is often detected when it has spread at the time of diagnosis, and the long-term prognosis is poor (Li et al., 2014). Therefore, early diagnosis of lung cancer is a realistic approach to reduce the mortality related with lung cancer. Although new instruments appear promising such as CT, PET and X-ray, which can not precisely realize early detection of lung cancer. The development of diagnostic tools is necessary for the early detection of lung cancer (Hassanein et al., 2012; Suzuki et al., 2010). Aberrant DNA hymethylation are involved in participating in the pathogenesis and progression of tumorigenesis and played an important role in non-small cell lung cancer (Gasche et al., 2012; Farkas et al., 2014; Suzuki et al., 2013; Chen et al., 2011; Daniunaite et al., 2011; Losi-Guembarovski et al., 2007; Missaoui et al., 2010; Zhang et al., 2011). Some scientific research have shown that RAR-β was absent in varying degrees in all kinds of malignant toumer (Chen et al., 2011; Daniunaite et al., 2011; Losi-Guembarovski et al., 2007; Wang et al., 2012; Zhang et al., 2011).

It is well known that decrease RAR-β expression is associated with the formation of a tumor and aberrant promoter methylation of the RAR-β gene is detected in NSCLC. However, genetic studies have not been attempted to identify the role of aberrant promoter methylation of the RAR-β gene that may be associated with NSCLC patients.

The present research is aimed at investigating the potential role of aberrant promoter methylation of the RAR-β gene in NSCLC risk and its effects on clinical characteristics in southern-central Chinese population.

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Materials and Methods

Study subjects

The study object included 167 newly diagnosed patients with NSCLC and 105 cancers-free controls. Final diagnoses of cases were confirmed by routine histopathological examination. All subjects were recruited between January 2010 and June 2013 between the Hunan provincial tumor hospital in Changsha (Changsha, China) and the Central Hospital of Zhuzhou City (Zhuzhou, China). At recruitment, written informed consents about the study were obtained from all the patients and controls. Each participant was then interviewed to collect information on demographic characteristics. The clinic pathological features of samples are shown in Table 1.

All study subjects provided written consents and were ethnic South-Central Chinese population Han, Each participant was interviewed to collect information on demographic characteristics. And the research protocol was approved by the Institutional Review Board of the hospital.

DNA extraction and bisulfite treatmen

Genomic DNA from peripheral blood lymphocytes was extracted using the standard kit-based method (Gentra Systems, Minneapolis, MN). Genomic DNA (0.5-1 μg) was treated with sodium bisulfite via the EZ DNA methylation-Gold kit (Zymo Research, USA). Bisulfite treatments changed unmethylated cytosines into uracils while leaving methylated cytosines unmodified. The bisulfite-modified DNA was used immediately for PCR or stored at -70°C.

Positive control for methylation

Lung cancer patient DNA was treated in vitro with excess SssI methyltransferase (New England Biolabs), to generate completely methylated DNA at all CpGs and was used as positive control for methylated alleles of each gene. DNA from healthy control sample was used as the control for unmethylated alleles. And Genomic DNA was treated with sodium bisulfite and stored at -70°C.

Methylation-specific PCR

Modified DNA was then used in MSP for the detection of the methylation status of RAR-β promoter. The primer sequences (Maruyama et al., 2002; Li et al., 2014), designed to amplify specifically methylated (M) or unmethylated (U) forms of the RAR-β promoter were: M1 (forward) 5'- TCGAGAACGCGAGCGATTC G-3'; M2 (reverse) 5'-GACCAATCCAACCGA AACGA-3'; U1 (forward) 5'-TTGAGAATGTG AGTGATTTGA-3'; U2 (reverse) 5'-AACCAAT CCAACCAAAACAA-3'. The PCR mixture contained 12.5μl TaqMix (Dongsheng, Biotech), 2μl of bisulfite-modified DNA, 1μl 10 pM each primer and 8.5 μl sterile water. The PCR profile was consisted of an initial denaturation at 94°C for 4 minutes, followed by 35 steps cycles of 94°C for 45 second, 62°C for 45 second, and 72°C for 60 second, and a final extension step of 72°C for 10 minutes. Water blank was used as a negative control. Both M and U primer set generated 169 bp products and the PCR products were analyzed in 2% TBE agarose gels.

Statistical analysis

Statistical analyses were performed using Statistical software SPSS13.0. The association between the methylation status of RAR-β gene and clinicopathological parameters was analyzed using a χ^2 test. The association between the methylation of RAR-β gene and NSCLC was determined using the logistic regression method to assess odds ratio (ORs) and 95% confidence intervals (95%CI).

Results

Characteristics of study subjects

The association with clinical features between lung cancer patient and healthy control sample are listed in Table 1. The factors of age and sex consumption had no significant difference between patients and controls. Conversely, there were a significant statistical between smoking and drinking status, which indicate that smoking and drinking are a risk factor for lung cancer in our study population.

RAR-β promoter methylation profile

Aberrant promoter methylation of the RAR-β gene was detected to 80.23% (134/167) of cases, and none of the 105 controls shown methylation in promoter methylation of the RAR-β gene. Table 2 shown distribution of methylation status in patients and controls and its association with risk of lung cancer. There was a significant statistical association of the promoter methylation of the RAR-β gene with lung cancer risk ($p<0.01$).

RAR-β promoter methylation profile and clinicopathological characteristics

Table 3 depicted the relationship between the methylation of the RAR-β gene and clinicopathological characteristics of lung cancers. Hypermethylation of the RAR-β gene was a significant statistical association with smoking status ($p=0.038$, $p<0.05$). No relationship was found between RAR-β gene methylation and other pathologic staging including clinical stage, cell type, gender and drinking ($p>0.05$). Those indicated cigarette smoking is a major risk factor of lung cancer.

Table 1. Demographic Characteristics of Cancer Patients and Controls

Characteristics	Cases (%)	Controls (%)	x^2-value	p-value
Total	167	105		
Age				
Mean+SD	58.2±16.7	51.6±13.2		
Gender				
Male (Ref)	104 (62.3)	58 (55.2)	1.326	0.052
Female	63 (37.7)	47 (44.8)		
Smoking status				
No (Ref)	44 (26.3)	82 (78.1)	69.428	0.000
Yes	123 (73.7)	23 (21.9)		
Drinking status				
No (Ref)	98 (58.54)	77 (73.33)	6.031	0.014
Yes	69 (41.9)	28 (26.67)		

[a] Ref, reference group

Table 2. Distribution of Methylation Status in Patients and Controls and Its Association with Risk of Lung Cancer

	Total	methylation (%)	unmethylation (%)	p-value
lungcancer (Ref)[a]	167	134 (80.23)	33 (19.77)	p<0.001
Controls	105	0	105 (100%)	

[a] Ref, reference group

RAR-β methylation of smoking lung cancer patients in pathological type and clinical stag

Table 4 was showed the association of RARβ methylation with pathological type and clinical stag in smoking lung cancer patients. No statistical difference was observed in the detection rate of RAR-β gene methylation between pathological type and clinical stag (p>0.05).

Discussion

Pathogenesis of lung cancer was a complicated biological process including multiple genetic and epigenetic changes (Lokk et al., 2012; Zhao et al., 2012). The role of the genetic mechanisms had become an increasing concern to global investigators in recent researches. DNA methylation profiles of lung cancer had suggested that the methylation was an important marker in genome modification, the aberrant promoter methylation of tumor suppressor genes was associated with mechanism in all kinds of cancer (Farkas et al., 2014; Suzuki et al., 2013; Chen et al., 2011; Daniunaite et al., 2011; Losi-Guembarovski et al., 2007). Although the range the methylation of some genes was low to high frequency (Hawes et al., 2010; Shaw et al., 2006), Methylation mechanism is still not very clear in process of cancers.

Several studies showed separately that methylation of CpG islands of RARβ genes had a significant role in the development of lung cancer (Jin et al., 2009; Zhao et al., 2012; Chung et al., 2011). A number of reports had proven that methylation of the RAR-β promoter affected numerous tumor-suppressing genes, which were subsequently silent and not expressed (Li et al., 2014). Other researches had indicated the abnormal methylation of RAR-β was associated with early pathogenesis of lung cancer, induced by correlative environmental factor exposure (Maruyama et al., 2002; Lokk et al., 2012; Shaw et al., 2006; Jin et al., 2009; Zhao et al., 2012). In the present study, the detection rate of methylation of the RAR-β gene exhibited significant differences between lung cancers as compared with control, there was a significant statistical association between lung cancer patients with controls. But no statistical differences were found in clinicopathological characteristics except for cigarette parameter with lung cancer patients, which was consistent with previous studies. These results suggest that RAR-β methylation and cigarette smoking were closely

Table 3. Frequencies of Methylation of RARB in 167cases of Lung Cancer

Variables	N	methylation (%)	unmethylation (%)	x^2-value	p-value	OR	95%CI
Gender							
Male (Ref)	104	79 (75.96)	25 (24.04)	3.182	0.074	0.46	0.193-1.094
Female	63	55 (87.30)	8 (12.70)				
Pathological type							
Squamous cell carinoma (Ref)	86	68 (79.07	18 (20.03)	0.153	0.696	0.859	0.400-1.844
Adenocarcinoma and other cell type	81	66 (81.48)	15 (18.52)				
Clinical stag							
I+II (Ref)	65	48 (73.85)	17 (26.15)	2.743	0.098	0.525	0.244-1.133
III+IV	102	86 (84.31)	16 (15.69)				
Cigarette							
No (Ref)	44	40 (90.90)	4 (9.10)	4.298	0.038	3.085	1.018-9.351
Yes	123	94 (76.42)	29 (23.58)				
Drinking status							
No (Ref)	98	81 (82.65)	17 (17.35)	0.871	0.351	1.438	0.669-3.093
Yes	69	53 (80.30)	16 (19.70)				

[a] Ref, reference group
[b] OR, odds ratio; CI, confidence interval.
[c] including the small cell, large cell, and mixed cell carcinomas or undifferentiated carcinomas

Table 4. RAR-β Methylation of Smoking Lung Cancer Patients in Pathological Type and Clinical Stage

Variables	N	methylation (%)	unmethylation (%)		x^2-value	p-value	OR	95%CI
	123	94 (76.42)	29 (23.58)					
Clinical stage								
I+II (Ref)	48	34 (70.83)	14 (29.07)	1.365	0.243	0.607	0.262-1.408	
III+IV	75	60 (80.00)	15 (20.00)					
Pathological type								
Squamous cell carinoma (Ref)	77	60 (77.92)	17 (22.08)	0.257	0.612	1.246	0.532-2.915	
Adenocarcinoma and other cell type	46	34 (73.91)	12 (26.09)					

[a] Ref, reference group
[b] OR, odds ratio; CI, confidence interval.
[c] including the small cell, large cell, and mixed cell carcinomas or undifferentiated carcinomas

related to the development process of NSCLC.

Tobacco smoking was responsible for substantial morbidity and mortality worldwide. And the methylation rate was closely correlated with the smoking, which might play a role in a variety of smoking-related phenomena (Breitling et al., 2011; Cheng et al., 2012; Wu et al., 2014). Previous science researches reported different results on the association analysis between RAR-β gene methylation and smoking status. A number of researchers had reported that the RAR-β gene was specifically targeted by carcinogens in cigarette smoke and the frequency of aberrant methylation of RAR-β gene increased in smokers (Zhao et al., 2012). In opposition, some authors failed to demonstrate a potential role of the RAR-β promoter methylation in lung carcinogenesis (Scesnaite A et al., 2012). In the present study, the detection rate of methylation of the RAR-β gene exhibited significant relationship between lung cancers and controls in smoking status, and was significantly higher in the non-smoking group that in the smoking group, Which increased 3.085 times greater risk of NSCLC compared with smoker. Those findings might suggest that tobacco smoking is a risk factor in the development process of NSCLC and tobacco plays a potential role in gene methylation.

RARβ methylation was analyzed in clinical stage and pathological type parameters with lung cancer patient, the results shown that no statistically significant difference was found. We were unable to conclude from this experiment whether RAR-β gene methylation could be used as an indicator of diagnosis of lung cancer on account of our small sample size. but the methylation of RAR-β gene rate of NSCLC is slightly higher in stages III+IV (80.0%) than in I+II (70.8%). And similar results were obtained for the methylation RAR-β gene between squamous cell carcinoma (77.9%) and other cell type lung cancer (73.9%). the frequency of methylation was increased gradually with the development of clinical stage in smoking lung cancer patients, and tobacco smoking played domain roles in NSCLC with hypermethylation of RAR-β gene prompter (Breitling et al., 2011; Scesnaite et al., 2012; Jin et al., 2010; Zeilinger et al., 2013; Wu et al., 2014). Although it was unclear that environmental factor underlie the targeting of specific genes promoters for hyperthyaltion, the animal experiment had shown the carcinogens can induce some genes methylated in mice (Jin et al., 2010; Scesnaite et al., 2012; Zeilinger S et al., 2013; Wu et al., 2014). Those data maybe suggest that tobacco play a potential role in RAR-β gene methylation in the early pathogenesis and process in lung cancer. The methylation of RAR-β gene prompter was considered to be a novel and promising marker of previous carcinogen exposure and cancer risk (Leong et al., 2011; Walter et al., 2014), especially in squamous cell carcinoma. Accordingly, it needs further work to increase sample size and understand the function of RAR-β gene at the molecular level.

In conclusion, we believed that RARβ methylation was associated with a higher susceptibility and has a prognostic significance in early stage to NSCLC, and may be a promising marker in early diagnosis with southern-central Chinese population Han. Although the results may be chance findings they nevertheless highlight the need to investigate interactions with tobacco smoke in studies on the promoter methylation.

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